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Changes in bradykinin and bradykinin B₂-receptor during estrous cycle of mouse

Padmasana Singh^a, Amitabh Krishna^{a,*}, and Rajagopala Sridaran^b

^aDepartment of Zoology, Banaras Hindu University, Varanasi 221005, India

^bDepartment of Physiology, Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310-1495, USA

Abstract

The aim of the study was to investigate changes in the abundance of bradykinin and bradykinin B₂-receptor in the ovary of mice during its estrous cycle. Changes in the abundance of bradykinin were correlated with changes in bradykinin B₂-receptor in order to determine the functional significance of this peptide for follicular development, ovulation and luteinization. Bradykinin immunoreactivity was mainly observed in the granulosa cells of antral follicles, especially around the oocytes and lining the antral cavity during proestrus and estrus phases of the cycle. Recently formed corpora lutea showed mild immunoreactivity for both bradykinin and bradykinin B₂-receptor. During diestrus 1 and diestrus 2, bradykinin and bradykinin B₂-receptor immunostaining was mainly found in the corpora lutea and mildly in the antral follicles. Immunoblot analysis for bradykinin and bradykinin B₂-receptor attained a peak during late evening in proestrus, which may be the time of the LH surge. Thereafter bradykinin and bradykinin B₂-receptor declined sharply during the estrus phase. When the concentration of bradykinin was correlated with bradykinin B₂-receptor throughout the estrous cycle, they showed strong positive correlation. Thus, this study indicates that the levels of bradykinin and bradykinin B₂-receptor both simultaneously regulate estrous cycle and are important components for the reproductive process.

Keywords

Bradykinin; Bradykinin B₂-receptor; Ovary; Estrous cycle; Mice

Introduction

Bradykinin is a physiologically and pharmacologically active peptide of the kinin group of proteins, consisting of nine amino acids. The kinin-kallikrein system consists of kininogens, kallikreins, kinins, kininases, kallikrein inhibitors and kinin receptors (Bhoola et al., 1992). Kininogens are primarily synthesized in the liver. However, kininogen-like substances are also found associated with several cell types, suggesting that kininogen production may be a local event (Proud et al., 1981). Bradykinin is formed by the proteolytic cleavage of its preferred kininogen precursor, high-molecular-weight kininogen (HMWK) by kallikrein enzyme. Another kinin, Lys-bradykinin, also known as kallidin, is produced via the action of an enzyme kallikrein on low molecular-weight kininogen (LMWK). Nearly all cells express kinin receptors, which mediate the activities of both bradykinin and kallidin. The half-life of bradykinin in plasma is less than 1 min. Bradykinin mediates a variety of responses in

tissues including smooth muscle contraction, vasodilation (Ludbrook et al., 1973), increased vascular permeability (Haddy et al., 1970), inflammation and pain (Bhoola et al., 1992).

Bradykinin is also known to play some specific roles in reproductive processes. Kinins have been detected in the follicular fluids of several species (Ramwell et al., 1969). Kihara et al. (2000) showed the presence of a component of bradykinin and the bradykinin-producing system in the porcine ovarian follicle, and suggested its role in early follicular development and ovulation. It has been demonstrated that bradykinin induces ovulation in perfused rat ovaries (Hellberg et al., 1991) and potentiates the action of LH during follicular rupture at the time of ovulation (Brännström and Hellberg, 1989). Also, a physiological role of bradykinin has been implicated in the LH surge (Shi et al., 1998). A recent study from our laboratory has demonstrated the presence of bradykinin and bradykinin B₂-receptor in various ovarian cells in several non-mammalian vertebrates (Singh et al., 2007).

Ohkura et al. (2003) detected bradykinin B₂-receptor mRNA in granulosa cells and corpora lutea at all stages of follicles in the mouse ovary, but the signal intensities differ from one follicle to another during proestrus. Therefore the aim of the present study was to localize the bradykinin and bradykinin B₂-receptor proteins in the ovary of mice during its estrous cycle and to determine the concentrations of the bradykinin and bradykinin B₂-receptor proteins during different phases of the estrous cycle which can be correlated with the ovulatory processes of the animal.

Materials and methods

Animals

All experiments were conducted in accordance with the principles and procedures approved by the Departmental Research Committee at Banaras Hindu University, Varanasi, India. Parkes strain mice were housed under controlled temperature ($24 \pm 2^\circ\text{C}$), humidity and illumination (lights on between 07.00 and 20.00 h). Food and water were available *ad libitum*. Adult female mice (10–12 weeks old) were used in this study. Estrous cycles were monitored through daily examination of vaginal cytology. Mice that exhibited at least two consecutive regular estrous cycles were selected for the study. Animals were sacrificed by decapitation under mild anesthesia (anesthetic ether) at 10.00, 12.00, 14.00 and 19.00 h on proestrus, 10.00 and 14.00 h on estrus, diestrus 1 and diestrus 2 of the estrous cycle. The ovaries were immediately dissected out and one side of the ovary was snap-frozen and kept at -40°C for immunoblotting analysis, whereas contralateral ovaries were fixed in Bouin's fixative for immunohistochemistry. The fixed tissues were dehydrated in ethanol, embedded in paraffin wax and sectioned at $5\ \mu\text{m}$.

Immunohistochemistry

The ovarian sections were processed through standard protocols of immunohistochemistry (Singh et al., 2007). The sections were deparaffinized, rehydrated in graded ethanol and endogenous peroxidase was quenched with 0.3% H₂O₂ in methanol. The sections were rinsed in 0.05 M Tris-HCl –0.15 M NaCl (TBS, pH 7.4). Background blocking was performed with 10% normal horse serum in PBS for 1 h. The tissue sections were then incubated for 1 h at room temperature either with bradykinin antibody (dilution 1:1500, Peninsula Labs Inc., San Carlos, CA, USA) or with bradykinin B₂-receptor antibody (dilution 1:25, BD Transduction Labs, San Jose, CA, USA) diluted in phosphate buffered saline (PBS). Following three rinses in PBS for 15 min, the slides were then incubated with secondary antibody. Detection system used was ABC universal staining kit (sc-PK-6200; Vector Labs., Burlingame, CA, USA). Peroxidase activity was revealed with 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) in 0.05

M Tris, pH 7.6 and 0.1% H₂O₂ for 10 min. Sections were rinsed in buffer. Nuclei were counterstained with Ehrlich's hematoxylin, prior to the sections being dehydrated and mounted with DPX. To test the specificity of the immunoreactions, in the control section the primary antiserum was replaced by: (1) 1% normal horse serum; (2) bradykinin antiserum with bradykinin antigen (200 ng/ml). For preadsorption, the antigens were added to diluted antisera (at the same dilution as used for localization), incubated overnight at 4 °C, centrifuged and then the supernatant was used.

Slot blot

The ovaries were collected during each phase of the estrous cycle and homogenized for protein extraction (Singh et al., 2007). Equal amounts of protein, as determined by Lowry's method (Lowry et al., 1951), were adjusted to equal volume with PBS and 10 µl of these samples were loaded on nitrocellulose membrane using Millipore slot blot apparatus. Non-specific sites were blocked with 5% non-fat dried milk in PBS, 0.02% Tween 20. Membranes were then incubated for 1 h at room temperature with bradykinin antibody (dilution 1:3000) in PBS. Membranes were washed in PBS-Tween 20 buffer and then incubated for 30 min with anti-rabbit IgG conjugated horseradish peroxidase (1:500, v/v). Immunoreactive bands were detected with enhanced chemiluminescence (ECL, Bio-Rad, Hercules, CA, USA). Experiments were performed in triplicate. Equal loading was confirmed with Ponceau S staining and β-actin expression. Validation of the slot blot for bradykinin was performed using serially diluted ovarian protein samples. The sensitivity of the blot was 0.1 µg.

Western blot

Equal amounts of protein as determined by Lowry's method (Lowry et al., 1951) were loaded on 10% SDS PAGE for electrophoresis. Separated proteins were then transferred on nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). Non-specific sites were blocked with 5% non-fat dried milk in PBS, 0.02% Tween 20. The membrane was then incubated for 1 h at room temperature with bradykinin B₂-receptor antibody (dilution 1:250) in PBS. Immunodetection was performed with secondary antibody conjugated with horseradish peroxidase (1:500 v/v). Finally, immunoreactive bands were detected with enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate. Equal loading was confirmed with Ponceau S staining and β-actin expression.

Densitometry

The relative intensities of different signals were quantified using a computer-assisted image-analysis system (Image J ver.1.36, NIH, Bethesda, MD, USA). The system was calibrated to have a constant parameter for illumination (intensity and area of light beam) throughout the experiment. Absorbance was expressed as normalized density value. Each measurement was repeated three times. The data are presented as the ±S.E.M. of the normalized density value of three blots of each peptide.

Statistics

Cyclic changes were analyzed by one-way ANOVA followed by Duncan's multiple range post hoc tests. Correlation studies were performed by using SPSS software 12 for window (SPSS Inc., Chicago, IL, USA) to compare the data from different phases of estrous cycle.

Results

Immunohistochemistry for bradykinin and bradykinin B₂-receptor in the ovaries of mice during the estrous cycle

Changes in the relative concentration of bradykinin and bradykinin B₂-receptor proteins expression in the ovaries of mice were studied during different phases of the estrous cycle using immunohistochemical analysis. The results are summarized in Fig. 1.

Bradykinin immunoreactivity was mainly observed in the granulosa cells of antral follicles during proestrus (Fig. 1A) and estrus phases (Fig. 1C) of the cycle. A moderate to intense immunoreactivity for bradykinin was observed in the granulosa cells located around the oocytes and lining the antral cavity. Recently formed corpora lutea showed mild immunoreactivity for bradykinin (Fig. 1C). Interstitial cells and thecal cells of antral follicles also showed moderate immunoreactivity for bradykinin. After the gonadotropin surge during late proestrus, bradykinin immunoreactivity increased in the granulosa cells and interstitial cells. Immunoreactivity declined during estrus and diestrus phases. During diestrus 1 (Fig. 1E) and diestrus 2 (Fig. 1G), bradykinin immunoreactivity was mainly found in the corpus luteum. Preadsorbed control for bradykinin showed no immunoreactivity (Fig. 1I).

Bradykinin B₂-receptor immunoreactivity was generally mild to moderate in the antral follicles during early proestrus. However, it increased during the gonadotropin surge on the afternoon of proestrus (Fig. 1B). Both follicles and corpora lutea showed mild immunoreactivity during estrus (Fig. 1D). A moderate immunoreactivity was found in the corpora lutea during diestrus 1 and mild immunostaining in the antral follicles (Fig. 1F) during diestrus 2 (Fig. 1H) phases.

Slot blot for bradykinin in the ovaries of mice during estrous cycle

The densitometric analysis of slot blot for bradykinin showed marked variation in the immunoreactivity between the ovaries of mice during different phases of estrous cycle. Intensity of the immunoblot for bradykinin showed a gradual increase in the expression level during proestrus and attained a peak during late evening in proestrus (19.00 h) and early estrus phases (Fig. 2). Bradykinin expression declined sharply during the afternoon of estrus, but subsequently increased again during the diestrus 1 phase, and declined once more during diestrus 2.

Western blot for bradykinin B₂-receptor in the ovaries of mice during the estrous cycle

Densitometric analysis of Western blot for bradykinin B₂-receptor showed two immunoreactive bands at ~42 and ~44 kDa (Fig. 3). Bradykinin B₂-receptor protein showed peak immunoreactivity during late evening in proestrus phase (19.00 h). Thereafter, the intensity of immunoreactivity of bradykinin B₂-receptor showed a gradual decline to attain a lowest concentration in early diestrus 1 phase. Thereafter, bradykinin B₂-receptor showed a moderate immunoreactivity during late diestrus 1 and diestrus 2 phases.

Correlation between ovarian bradykinin with bradykinin B₂-receptor during the estrous cycle

Relative density values of the immunoblots for bradykinin and bradykinin B₂-receptor are based on analysis of total ovarian extracts with specific antibodies. Changes in the ovarian concentration of bradykinin were correlated with the ovarian concentration of bradykinin B₂-receptor during different phases of estrous cycle using computer based SPSS program (IBM SPSS Inc., Chicago, IL, USA). Bradykinin showed positive correlation with both

upper band ($r = 0.420$; $P < 0.05$) and lower band ($r = 0.403$; $P < 0.05$) of bradykinin B₂-receptor.

Discussion

Previous studies have shown a bradykinin generating system in the ovary of rat (Brann et al., 2002) and bradykinin and its metabolites in the follicular fluid of the pig (Kihara et al., 2000). Ohkura et al. (2003) has shown the presence of bradykinin B₂-receptor mRNA in the ovary of immature mice treated with human chorionic gonadotropin (hCG) and in the ovary of adult mice during its estrous cycle. They also demonstrated bradykinin B₂-receptor by Western blotting, but failed to show it by immunohistochemistry. On the basis of their results they hypothesized that the receptor protein is present on the plasma membrane of the granulosa cells and in the corpus luteum. In the present study we showed localization of bradykinin and bradykinin B₂-receptor proteins by immunohistochemistry and immunoblot analysis in the ovary of mice. Our results further showed that there is a significant change in the relative abundance of these proteins during the mouse estrous cycle. Both bradykinin and bradykinin B₂-receptor showed strong immunoreactivity, mainly in the granulosa cells, and moderate immunoreactivity in the theca cells of the antral follicles during proestrus and estrus phases. These observations are in agreement with the previous observation showing the localization of bradykinin B₂-receptor mRNA in the granulosa cells of the antral follicles (Ohkura et al., 2003). This study showed relatively less abundance of bradykinin and bradykinin B₂-receptor immunoreactivity during early proestrus, but the concentration of bradykinin and its receptor immunoreactivity increased significantly on the evening of proestrus, which is the time of LH surge. Based on this result, we conclude that expression of both bradykinin and its receptor increases during the LH surge and are involved in cascades of reactions during ovulation. The ovulatory process includes: contraction of the follicle wall, which may help expel the intrafollicular oocytes, perifollicular vascular alterations leading to follicle wall ischemia and increased permeability of vessels, proteolytic enzyme activity, which disrupts collagen fibers in the follicle wall, and an inflammatory reaction involving several hormonal components, prostaglandins, histamine, bradykinin and superoxide anions. It has been demonstrated that kinin producing activity increases during ovulation (Smith and Perks, 1983; Espey et al., 1989; Gao et al., 1992). Bradykinin-induced ovulation is accompanied by production of both PGF₂ α and PGI₂. A previous study also showed an increase in bradykinin B₂-receptor levels 6 h after hCG treatment in mice (Ohkura et al., 2003). Interestingly, bradykinin treatment of isolated granulosa cells was found to induce gene expression of some matrix metalloproteinases, which may be involved in follicle rupture during ovulation (Kimura et al., 2001). These findings may provide a clue to explain the mechanisms of follicle rupture during ovulation induced by bradykinin. Thus, the present study, together with the earlier studies, strongly suggests that the bradykinin system plays a role in the ovulatory process in rodents.

The immunostaining for bradykinin and bradykinin B₂-receptor subsequently shifted from follicular cells to the luteal cells during diestrus phases. A significant increase in the concentration of bradykinin and its receptor during diestrus 1 coincides with the activity of the corpus luteum. Bradykinin concentration decreases at late diestrus 2 phase with a decline in the corpus luteum activity and initiation of new wave of follicular growth. Also there is decreased abundance of bradykinin and its receptor in the ovary that has preantral and early antral follicles. This is in agreement with the previous finding of strong bradykinin B₂-receptor mRNA expression in the late antral follicles and weak expression in the smaller follicles (Ohkura et al., 2003).

In summary, the highest abundance of bradykinin was detected during the peak abundance of bradykinin B₂-receptor at the period around ovulation, and also the concentration of

bradykinin showed positive correlation with bradykinin B₂-receptor in the ovary of mice during its estrous cycle. This suggests that bradykinin and bradykinin B₂-receptor are co-regulated. It also raises the possibility that bradykinin may be regulating its own receptor in the ovary of mouse. It is further hypothesized that the mouse ovary possesses a bradykinin system, which may be involved in the regulation of follicular development and ovulation.

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Fig. 1.

Immunohistochemical localization of bradykinin and bradykinin B₂-receptor proteins in the ovaries of mice. (A) A portion of the ovary showing intense bradykinin immunoreactivity in the granulosa cells (GCs), with mild immunoreactivity in the theca cells (Tcs) of the antral follicles during proestrus; (B) strong bradykinin B₂-receptor immunoreactivity in the granulosa cells (GCs), but mild immunoreactivity in the theca cells (Tcs) of the antral follicles during proestrus; (C) a portion of a newly formed corpus luteum during estrus phase showing mild bradykinin immunoreactivity; (D) mild immunoreactivity for bradykinin B₂-receptor in the follicle during estrus phase; (E) moderate immunoreactivity for bradykinin in the corpus luteum (CL) during diestrus 1; (F) moderate immunoreactivity for bradykinin B₂-receptor in the corpus luteum (CL) during diestrus 1; (G) moderate immunoreactivity for bradykinin in the corpus luteum (CL) and mild in the follicle during diestrus 2; (H) moderate immunoreactivity for bradykinin B₂-receptor in the corpus luteum and mild in the granulosa cells (GCs) of the follicle during diestrus 2; (I) absence of immunoreactivity in preadsorbed control for bradykinin.

Fig. 2. Slot blot for bradykinin (A) in the ovaries of mice during its estrous cycle. Densitometric analysis of the blot is given in bar graph (B). Values are represented as mean $\pm$ S.E.M. of five samples. Bar bearing different superscript (a–g) indicates significant difference between the mean values ($P < 0.05$). (1) proestrus 10.00 h; (2) proestrus 12.00 h; (3) proestrus 14.00 h; (4) proestrus 19.00 h; (5) estrus 10.00 h; (6) estrus 14.00 h; (7) diestrus 1 10.00 h; (8) diestrus 1 14.00 h; (9) diestrus 2 10.00 h; (10) diestrus 2 14.00 h.

Fig. 3.

Western blot for bradykinin B₂-receptor (A) in the ovaries of mice during its estrous cycle. Densitometric analysis of the blot is given in bar graph (B). Values are represented as mean $\pm$ S.E.M. of five samples. Bar bearing different superscript (a–g) indicates significant difference between the mean values ($P < 0.05$). (1) proestrus 10.00 h; (2) proestrus 12.00 h; (3) proestrus 14.00 h; (4) proestrus 19.00 h; (5) estrus 10.00 h; (6) estrus 14.00 h; (7) diestrus 1 10.00 h; (8) diestrus 1 14.00 h; (9) diestrus 2 10.00 h; (10) diestrus 2 14.00 h.